A class B scavenger receptor mediates the cellular uptake of carotenoids in *Drosophila*

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Carotenoids are currently being intensely investigated regarding their potential to lower the risk of chronic disease and vitamin A deficiency. Invertebrate models in which vitamin A deficiency is not lethal allow the isolation of blind but viable mutants affected in the pathway leading from dietary carotenoids to vitamin A. Using a mutant in one of these model systems, *Drosophila*, the vitamin A-forming enzyme has recently been molecularly identified. We now show that the molecular basis for the blindness of a different *Drosophila* mutant, *ninaD*, is a defect in the cellular uptake of carotenoids. The *ninaD* gene encodes a class B scavenger receptor essential for the formation of the visual chromophore. A loss of this function results in a carotenoid-free and thus vitamin A-deficient phenotype. Our investigations provide molecular insight into how carotenoids may be distributed into cells of target tissues in animals and indicate a crucial role of class B scavenger receptors rendering dietary carotenoids available for subsequent cell metabolism, as needed for their various physiological functions.

Carotenoids are C_{40} isoprenoids synthesized in plants, certain fungi and bacteria with characteristic molecular structures and properties responsible for light absorption as well as for the inactivation of aggressive radicals (reviewed in ref. 1). Among the various classes of pigments found in nature, the diverse family of yellow to red-colored carotenoids is the most widespread, with important functions not only in carotenoid-producing organisms. Some animals use dietary carotenoids for coloration. Well known examples are the feathers of flamingos and the red color of salmon. Because of their antioxidative properties, beneficial effects have also been reported for carotenoids in the prevention of coronary heart diseases, certain kinds of cancer, and age-related macular degeneration in humans (reviewed in ref. 2).

Most important, certain carotenoids are the precursors (provitamins) for the formation of vitamin A in animals. This vitamin is needed for vision in the entire animal kingdom. The visual pigments (rhodopsins) of animals are composed of a retinoid chromophore (vitamin A derivative) bound to a protein moiety (opsin) embedded in the photoreceptor membranes (3, 4). Light activation of the visual pigments triggers a G protein-coupled receptor cascade leading to changes in the permeability of the photoreceptor cell membranes. Besides being crucial for vision, in vertebrates vitamin A is also important in development and cellular differentiation processes. Here, the vitamin A derivative retinoic acid, together with its nuclear receptors, is involved in the regulation of diverse target genes; consequently, complete vitamin A deficiency leads to early embryonic death (5).

To become biologically active, dietary carotenoids must first be absorbed, then delivered to the site of action in the body and, in the case of provitamin A function, metabolically converted. Despite the general importance of carotenoids in animals, their metabolism is still poorly understood (6). Invertebrates like *Drosophila* represent excellent models for the genetic dissection of the pathway leading from dietary carotenoids to vitamin A. Here, this vitamin is only needed for vision; therefore, its deficiency has no fatal consequences. Among the various *Drosophila* mutants affected in their visual performance (4), the two mutants *ninaB* and *ninaD* lack the visual chromophore of the fly, 3-hydroxyretinal, when raised on standard media with carotenoids as the sole source for vitamin A formation (7). By analyzing the molecular basis of the blindness of *ninaB* mutants, we already showed that the phenotype is caused by mutations in a gene coding a carotene-15,15′-oxygenase and molecularly identified the key enzyme for carotenoid conversion to vitamin A in animals (8, 9). By sequence identity, orthologs to this insect gene were cloned from several vertebrate species including man, showing that the enzymes catalyzing vitamin A formation are evolutionarily well conserved (10–13). In *Drosophila*, mRNA expression of *ninaB* was exclusively found in the head, in agreement with retinoids being restricted in their distribution to the eyes (8, 14). In vertebrates (with vitamin A needed also for cellular differentiation processes), the vitamin A-forming enzyme is expressed in a variety of different tissues including reproductive tissues and the eyes (10, 12, 13). After dietary absorption, carotenoids must be distributed to these tissues to be converted to vitamin A.

In the second chromophore-less *Drosophila* mutant, *ninaD*, the carotenoid content was shown to be specifically and significantly altered compared with wild-type (wt) flies and was ineffective at mediating visual pigment synthesis (14). This phenotype is presumably caused by a defect in the body distribution of dietary carotenoids and makes the *ninaD* gene an interesting candidate for a molecular player necessary for these transport processes.

Here, we report on the identification of the *ninaD* gene encoding a scavenger receptor with significant sequence identity to the mammalian class B scavenger receptors, SR-BI and CD36. In $ninaD^{P245}$ flies, there is a nonsense mutation in the gene coding this receptor, thus abolishing its function. By *P* element-mediated transformation with a *wt ninaD* allele by using the UAS/GAL4 system, we could rescue the blind phenotype of $ninaD^{P245}$ flies. Heat shock (hs)-induced *wt* transgene expression resulted in carotenoid accumulation as judged by quantitative HPLC analyses and subsequent restoration of visual pigments. The identification of this scavenger receptor as being essential for the cellular uptake of carotenoids in *Drosophila* promises to elucidate new aspects of class B scavenger receptor functions with respect to carotenoid and vitamin A metabolism also in mammals.

Materials and Methods

***Drosophila* Strains, Construction of Transgenic Flies, Crosses, and Heat Shock Treatment.** Fly strains were raised on standard corn medium at 22°C. The $ninaD^{P245}$ strain had the genotype *w;* $ninaD^{P245}/CyO;$ +/+. As *wt* control, yellow white flies were used. To obtain a UAS-$ninaD^{(wt)}$ fly strain, we cloned the long splicing variant of the wild-type *ninaD* cDNA into the vector

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Abbreviations: wt, wild type; RT, reverse transcription; hs, heat shock; RACE, rapid amplification of cDNA ends.

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pUAST and transformed yellow white flies with the resulting plasmid (15). By crosses of the resulting transgenic flies with *ninaD^{P245}* and balancer flies, we maintained flies with the genotype *w;ninaD^{P245}/CyO;UAS-ninaD$^{(wt)}$/UAS-ninaD$^{(wt)}$*. For heat shock rescue, we crossed these flies with *w;ninaD^{P245}/CyO;hs-GAL4/hs-GAL4* flies and obtained *w;ninaD^{P245}/ninaD^{P245};hs-GAL4/UAS-ninaD$^{(wt)}$* flies. As controls, we used *w;ninaD^{P245}/ninaD^{P245};hs-GAL4/+* and *w;ninaD^{P245}/ninaD^{P245};UAS-ninaD$^{(wt)}$/+* flies and *w;ninaD^{P245}/CyO;+/+* flies, respectively. Heat shock was for 1 h at 37°C. The flies were analyzed 72 h later. For the dietary rescues of the *ninaD^{P245}* flies, the corn medium (total carotenoid content 1 pmol/mg) was supplemented with retinal (100 pmol/mg corn medium) or carotenoids (100 pmol/mg corn medium), both purchased from Sigma. To place the UAS-*ninaD$^{(wt)}$* transgene under the control of the ninaE-GAL4 driver, we crossed ninaE-GAL4 flies having the genotype *w;ninaE-GAL4/ninaE-GAL4;TM2/MKRS* with UAS-*ninaD$^{(wt)}$* flies of the genotype *w;CyO/wg^{Sp-1};UAS-ninaD$^{(wt)}$/UAS-ninaD$^{(wt)}$* and analyzed the offspring.

PCR Analyses To Test for a Mutation in CG5750. To test for a mutation in the genomic sequence of CG5750 in *ninaD^{P245}* flies, we isolated genomic DNA from 20 flies and performed PCR analyses using *Taq* polymerase (Amersham Pharmacia) and the following sets of oligonucleotide primers: 5′-CCAAACCGAGCTGATTACCAC-3′ and 5′-CCCAGAACCAAGTTCTTCTG-3′, 5′-GAAGATGGATCTTGAGTGGC-3′ and 5′-CCTTCAACGGCACTCCCATC-3′, 5′-CAACCGGAGCCACTGACCTAG-3′ and 5′-GATAAAAGAGTTGGGAGCCAG-3′, 5′-GCCTCAGCCTGGCCAGCC-3′ and 5′-CGGGCACTCGCGATTCTTG-3′, 5′-AAGGCGGCGCCACCCATATG-3′ and 5′-GGTTTTGGAGACTGGGCAG-3′. For the determination of the DNA sequences, the PCR products were directly sequenced.

Reverse Transcription (RT)-PCR and PCR Analyses To Check for Fusion of Two Genes. Reverse transcription was performed with mRNA isolated from total RNA of 20 *wt* flies by using an Oligotex mRNA mini kit (Qiagen, Valencia, CA), an oligo(dT)$_{17}$ primer, and Superscript reverse transcriptase (Invitrogen). PCR analyses were performed with the resulting first strain cDNA, and genomic DNA were isolated from 20 *wt* flies, respectively. The following sets of oligonucleotide primers were used: 5′-CGAGTCGGATCACTTTGCCTG-3′ and 5′-CGGGCACTCGCGATTCTTG-3′; 5′-CGAGTCGGATCACTTTGCCTG-3′ and 5′-CCTTCAACGGCACTCCCATC-3′; and 5′-AAGGCGGCGCCACCCATATG-3′ and 5′-CGGGCACTCGCGATTCTTG-3′. PCR conditions were 94°C for 2 min, 32 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 80 s, and finally 72°C for 10 min.

3′- and 5′-Rapid Amplification of cDNA Ends (RACE)-PCR. RACE-PCRs (3′ and 5′) were performed with mRNA of *wt* flies by using a FirstChoice RLM-RACE kit (Ambion, Austin, TX). The following gene-specific primers were used: 5′-GAAGATGGATCTTGAGTGGC-3′ and 5′-CAACCGGAGCCACTGACCTAG-3′ for the 3′-RACE and 5′-GAATCACATCCGTGTGCTGCTC-3′ and 5′-CGGCCTGGCCATACCACTTC-3′ for the 5′-RACE. PCR conditions were 94°C for 2 min, 32 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 100 s, and finally 72°C for 10 min. PCR products were subcloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced.

Northern Blot Analyses. Northern Blot analyses were carried out according to the DIG Application Manual for Filter Hybridization (Roche Diagnostics). To obtain the riboprobe, PCR was performed using the oligonucleotide primers 5′-GAAGATGGATCTTGAGTGGC-3′ and 5′-CCTTCAACGGCACTCCCATC-3. The PCR product was subcloned into the vector pCR2.1-TOPO (Invitrogen), and the antisense riboprobe was synthesized with T7 RNA polymerase by using a DIG-RNA labeling kit (Roche Diagnostics). RNA (4 μg total) and mRNA corresponding to 4 μg of total RNA, respectively, were used per lane of the 1.2% formaldehyde gel.

Analyses of the Major Rhodopsin of Flies. Proteins from three fly heads were extracted in 6% β-mercaptoethanol/6% SDS/20% glycerol/0.6% bromphenol blue, separated by SDS/PAGE on a 12% gel, and transferred onto a nitrocellulose membrane (Amersham Pharmacia). Rh1 was determined using a polyclonal antibody (16) and the enhanced chemiluminescence system (Amersham Pharmacia).

Extraction of Carotenoids and HPLC Analyses. Flies were homogenized in 200 μl of 2M NH_2OH with a loose fitting potter. Then 400 μl of methanol and 600 μl of acetone were added. Lipophilic compounds were extracted three times with 500 μl of petroleum ether. The collected organic phases were dried under a stream of N_2 and dissolved in the HPLC solvent (diethyl ether/*n*-hexane/ethanol, 79/20/1). HPLC systems were as described (10).

Lipid Analyses and GC/MS Analyses. Lipid analyses were carried out using Merckotest 3321 Total Lipids (Merck). For each experiment, 70 flies were homogenized in 0.9% NaCl solution with a loose fitting potter. For GC/MS analyses, 10 flies (5 female and 5 male flies) were homogenized in 500 μl of 50 mM tricine/100 mM NaCl, pH 7.5, and extracted three times with 500 μl of chloroform/methanol. After saponification and silanization, fatty acids were subjected to GC/MS analyses using the conditions described (10).

***In Situ* Hybridization of Embryos.** Embryo dechorionation, devitellination, and fixation was carried out according to ref. 17. *In situ* hybridization was performed as described (18). The riboprobe was the same as used in Northern blot analyses. Staging of embryos was performed according to ref. 19.

Results

Characterization of the Phenotype of *ninaD^{P245}* Flies. Because the carotenoid composition of *ninaD^{P245}* flies had yet not been analyzed, we first addressed this question. We raised a heterozygous, balanced *ninaD^{P245}* stock on standard corn medium containing zeaxanthin, lutein, cryptoxanthin, and β-carotene with a molar composition of approximately 9:4:2:1 (all of which exert provitamin A activity in flies) and collected homozygous *ninaD^{P245}* flies. As determined by HPLC analyses, the carotenoid contents of both the trunks and heads of adult homozygous *ninaD^{P245}* flies were highly reduced compared with controls (Fig. 1 *A* and *B*). To judge more directly the visual pigment content of *ninaD^{P245}* flies, we determined the amount and maturation status of the major rhodopsin Rh1 of the flies by Western blot analysis. It has been previously shown that in *ninaD^{P246}* flies, the major opsin Rh1 is only found in its immature glycosylated form, most probably because of the absence of the visual chromophore (20). As expected, in contrast to controls, Rh1 also existed only in its immature form in *ninaD^{P245}* flies (Fig. 1*C*). Next, in rescue experiments we supplemented the corn medium with vitamin A (retinal) and with additional β-carotene and zeaxanthin and measured Rh1 maturation. Feeding *ninaD^{P245}* flies retinal led to Rh1 maturation, showing that preformed vitamin A can compensate the blockade in carotenoid utilization. Additionally, supplementation with very high amounts of β-carotene but not zeaxanthin resulted in Rh1 maturation. Thus, the *ninaD^{P245}* mutation seems to differentially affect the utilization of β-carotene and zeaxanthin. As shown above, normal corn medium already contained significant amounts of β-carotene. Hence,

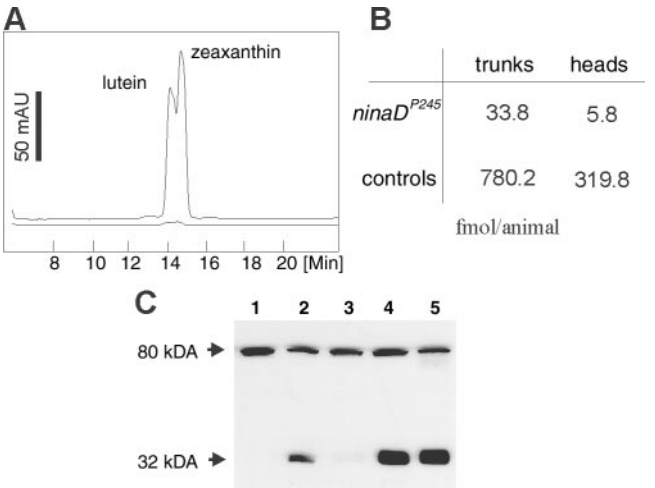

Fig. 1. The carotenoid and visual pigment content of *ninaD*^{P245} flies is drastically reduced. (*A*) HPLC analyses of the lipohilic compounds of the fly's head are shown. The two major carotenoids were lutein and zeaxanthin. The upper lane gives the HPLC profile at 450 nm of an extract from heterozygous *ninaD*^{P245}, the lower lane of an extract of homozygous *ninaD*^{P245}; each extract was yielded from the heads of 3,000 flies. (*B*) Total carotenoid contents of heads and trunks of homozygous *ninaD*^{P245} flies and heterozygous controls. The values give the average of 3,000 flies. (*C*) Western blot analyses showing the content and maturation state of the major rhodopsin Rh1 of the flies. Immature Rh1 migrates as a dimer at 80 kDa, mature Rh1 at 32 kDa. Lane 1, homozygous *ninaD*^{P245} raised on standard corn medium; lane 2, homozygous *ninaD*^{P245} raised on standard corn medium supplemented with β-carotene; lane 3, homozygous *ninaD*^{P245} raised on standard corn medium supplemented with zeaxanthin; lane 4, homozygous *ninaD*^{P245} raised on standard corn medium supplemented with retinal; lane 5, heterozygous *ninaD*^{P245} controls.

Rh1 maturation upon feeding additional β-carotene may be explainable by a passive diffusion when very high amounts of this pure hydrocarbon are available. In conclusion, these analyses indicated that the *ninaD* mutation interferes with the absorption and body distribution of carotenoids and therefore resulted in vitamin A deficiency.

Identification of the *ninaD* Gene. The *ninaD* mutation has been cytologically mapped to the genomic position 36E-F on chromosome 2 close to the fly's arrestin1 gene (*arr1*) (7). The Arr1 protein is involved in the regeneration of *meta*-rhodopsin in the visual cycle, but it could be shown that this protein is not defective in *ninaD* flies (21). Therefore, we searched the genomic region next to *arr1* and focused our attention on the predicted gene CG5750 (Flybase). The deduced amino acid sequence of CG5750 shares significant sequence similarity to the mammalian class B scavenger receptors *CD36* and *SR-BI* (22). SR-BI mediates bidirectional flux of cholesterol from lipoproteins (high and low density lipoproteins) to target cells in mammals (23, 24). Because carotenoids are isoprenoids like cholesterol, known to be transported in insects by lipophorins, which are structurally related to the mammalian lipoprotein classes (25), we wondered whether a mutation in this gene might be the cause for the blockade in carotenoid utilization of *ninaD*^{P245} flies. To test this possibility, we designed several sets of oligonucleotide primers overlapping the entire genomic sequence of CG5750 and performed PCR on genomic DNA isolated from *ninaD*^{P245} flies. By direct sequencing of the PCR products, we found a nonsense mutation in the deduced coding region (Fig. 2*A*). Thus, we supposed that CG5750 encodes the *ninaD* gene.

Molecular Structure of the Putative *ninaD* Gene. By computer prediction, CG5750 encodes a protein 861 aa in length (Flybase), approximately double the size of the known type B scavenger receptors. On closer inspection, it turned out that the amino acid sequence consists of two recurring parts, both sharing significant overall similarity to class B scavenger receptors. The most plausible explanation seemed to be that the algorithm of the computer program incorrectly fused the coding regions of two individual genes. This hypothesis could be confirmed by RT-PCR analysis, by which no product was obtained with a pair of primers spanning the predicted coding region (Fig. 2*B*). To analyze the correct molecular structure and to clone the cDNA of the putative *ninaD* gene defined by the nonsense mutation, we performed 3′- and 5′-RACE-PCR experiments. We obtained two different products for the 3′-end (Fig. 2*C*) and a single product for the 5′-end. Sequencing of the RACE-PCR products revealed the existence of two different splicing variants for the 3′-end. By a Northern blot we confirmed this result (Fig. 2 *D* and *E*). This analysis also showed that the mRNA levels of the putative *ninaD* gene were significantly reduced in heterozygous *ninaD*^{P245} flies and were hardly detectable in homozygous *ninaD*^{P245} flies. This decrease is probably caused by rapid degradation of the mRNA of the *ninaD*^{P245} allele due to the nonsense mutation, as previously reported for mutated genes (26). The longer mRNA of the putative *ninaD* gene was 1,736 nucleotides long, coding a protein of 513 aa residues; the second mRNA lacks the last two exons, thus coding for an identical but C-terminally shortened protein of 415 residues. The deduced amino acid sequences shared overall similarities to the *Drosophila* CD36-like scavenger receptor proteins *croquemort* and *emp* (27, 28) and to mammalian class B scavenger receptors. An N-terminal stretch of hydrophobic residues (residues 15–36) probably serves as an uncleaved signal sequence (Fig. 2*F*). There are also five putative N-glycosylation sites (residues 72, 110, 217, 248, and 261) near the C terminus five clustered cysteine residues (residues 286, 326, 328, 337, and 348) and a putative transmembrane domain (residues 457–477). Comparison with human CD36 and SR-BI reveals several conserved cysteine, glycine, and proline residues and 22.5 and 24% sequence identity, respectively. Both NinaD and SR-BI possess a C-terminal extension compared with CD36. Interestingly, a threonine found in a putative protein kinase C consensus sequence (GPYTYR) in CD36 (residues 89–94) is replaced by a valine (GPYVYR) in NinaD (residues 92–97), as in SR-BI. This phosphorylation site has been proposed to be a regulatory domain in CD36, whereas replacement of the threonine by a valine residue as in SR-BI and NinaD may predict constitutive, nonregulated binding activity (29).

Rescue of *ninaD*^{P245} Flies by *P* Element-Mediated Transformation with a cDNA Coding the Wild-Type Allele. To demonstrate that this scavenger receptor is essential for carotenoid utilization and thus is responsible for the blindness of *ninaD* flies, we established an *in vivo* system by using *P* element-mediated transformation and the UAS/GAL4 expression system (15). For this purpose, we transformed flies with a *wt* cDNA (representing the longer splice variant) in the vector pUAST, resulting in a fly strain with *UAS-ninaD*$^{(wt)}$ on chromosome 3. By further crosses, homozygous *ninaD*^{P245} flies carrying the *UAS-ninaD*$^{(wt)}$ and *hs*-GAL4 transgenes on chromosome 3 were generated and tested for the rescue of the blind *ninaD* phenotype by heat shock-induced transgene expression. To assay for this rescue, we analyzed again the maturation of the fly's major rhodopsin Rh1. In the *ninaD*^{P245} flies carrying the *UAS-ninaD*$^{(wt)}$ and *hs*-GAL4 transgenes without heat shock treatment, Rh1 was only found in its immature form. Heat shock-induced *ninaD*$^{(wt)}$ expression resulted in Rh1 maturation, accompanied by a reduction in its molecular mass to 32 kDa (Fig. 3*A*). Furthermore, to show directly that the *ninaD*$^{(wt)}$ transgene expression resulted in carotenoid uptake in *ninaD* flies, we determined the content and

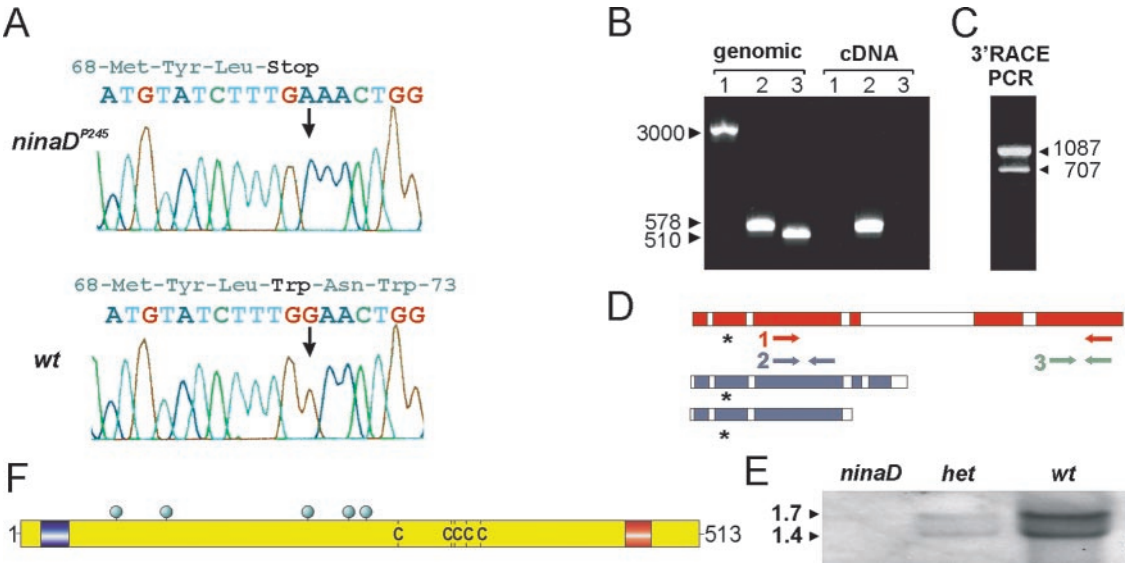

Fig. 2. A nonsense mutation is found in the two splicing variants of the *ninaD* gene. (*A*) We performed PCR analyses with genomic DNA from homozygous *ninaD*^{P245} and wt flies by using several sets of oligonucleotide primers overlapping the entire genomic region of the predicted gene CG5750. Sequencing of the PCR products revealed a base pair exchange (G to A) in homozygous *ninaD*^{P245} flies, resulting in a stop codon at position 71 of the predicted amino acid sequence of CG5750. (*B*) To judge whether CG5750 consists of two genes, we performed PCR and RT-PCR analyses on genomic DNA and mRNA preparations from *wt* flies by using three different primer pairs. Primer pair 1 spanned exons 3 to 6, primer pair 2 was specific for exon 3, and primer pair 3 was specific for exon 6 of the predicted gene CG5750 (see *D*). Agarose gel electrophoresis revealed PCR products with genomic DNA for all three primer pairs; an RT-PCR product could only be obtained with primer pair 2. (*C*) 3′-RLM-RACE-PCR with a 5′ oligonucleotide primer specific for exon 3 of the predicted gene CG5750 resulted in two different products, showing the existence of two splicing variants. (*D*) The predicted exon/intron structure of CG5750 (red) and the determined exon/intron structures of the two splicing variants of the putative *ninaD* gene (blue). The longer splicing variant consists of five exons, the fifth not primarily predicted in GC5750, with 164, 303, 812, 91, and 366 bases, respectively. The shorter variant includes only the first three exons. Coding regions are colored, and the position of the point mutation found in the putative *ninaD*^{P245} allele is indicated by asterisks. (*E*) Northern blot analyses with mRNA preparations obtained from *wt*, heterozygous (*het*), and homozygous (*hom*) *ninaD*^{P245} flies confirmed the existence of the two splicing variants of the putative *ninaD* gene. In heterozygous *ninaD*^{P245} flies, the levels of the putative *ninaD* mRNAs were strongly reduced compared with *wt* flies; in homozygous *ninaD*^{P245} flies (*ninaD*) they were hardly detectable. (*F*) Primary structure of the putative NinaD protein. The positions of the N-terminal and C-terminal transmembrane domains are indicated in blue and red; the positions of the putative N-glycosylation sites are indicated with green-filled circles, and clustered cysteine residues are indicated by a ''C.''

composition of carotenoids. By quantitative HPLC analyses, we found a significant increase in zeaxanthin and lutein (Fig. 3*B*). Thus, the *ninaD* gene encodes a class B scavenger receptor possessing *in vivo* an essential role in mediating the cellular uptake of carotenoids for the synthesis of the visual chromophore.

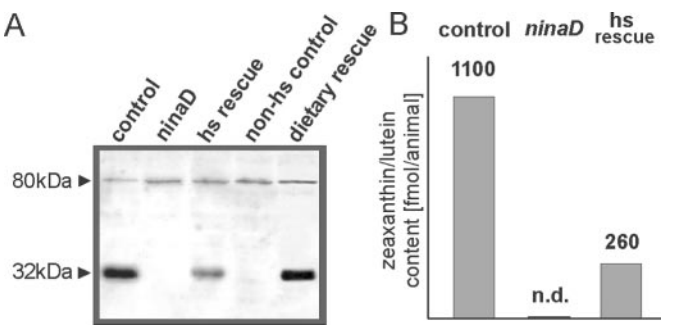

Fig. 3. Expression of a *ninaD*$^{(wt)}$ transgene restores the vitamin A-deficient phenotype of *ninaD*^{P245} flies. Adult homozygous *ninaD*^{P245} flies carrying a UAS-*ninaD*$^{(wt)}$ transgene expressed under the control of a hs-GAL4 driver were compared with heterozygous and non-heat-shocked homozygous control flies. The flies used had the following genotypes: control, *w*; *ninaD*^{P245}/*CyO*; +/+; ninaD, *w*; *ninaD*^{P245}/*ninaD*^{P245}; +/+; hs rescue and non-hs control, *w*; *ninaD*^{P245}/*ninaD*^{P245}; *hs-GAL4*/*UAS-ninaD*$^{(wt)}$. hs rescue flies were heat-shocked for 1 h at 37°C and analyzed 72 h later. For the dietary rescue of *ninaD* flies, the corn medium was supplemented with retinal for 3 days. (*A*) Protein extracts were subjected to Western blot analysis using an anti-Rh1 antibody. Mature Rh1 migrated at 32 kDa, immature Rh1 as a dimer at 80 kDa. (*B*) Carotenoid contents of the whole flies were determined by HPLC analyses, and for quantification, known amounts of authentic carotenoid standards were used. The values give the average of 63 flies per experiment. n.d., not detectable.

Total Lipid Composition of *ninaD*^{P245} Flies. The mammalian class B scavenger receptors are multifunctional, mediating the uptake of lipids such as long chain fatty acids and sterols from lipoproteins. Therefore, to ask whether the *ninaD* mutation might generally interfere with the lipid metabolism in the fly, we determined the total lipid content in *ninaD*^{P245} flies. By this analysis, no differences in the total amounts of lipids were detectable compared with control flies. After saponification and silanylation of fatty acids, we also analyzed the profile of these compounds by GC/MS analysis. Again, we found no differences in *ninaD*^{P245} flies compared with controls. The qualitative composition of fatty acids was similar to that previously reported in *Drosophila* (30) with the major constituents 14:0, 16:0, 16:1, 18:0, 18:1, and 18:2. Thus, we conclude that the NinaD scavenger receptor is crucial for carotenoid uptake but evidently dispensable for the lipid metabolism as a whole.

Temporal Expression Pattern of the *ninaD* Gene. Having established this new function in carotenoid uptake of a scavenger receptor, we determined the temporal aspects of the *ninaD* gene expression. We first analyzed different embryonic stages by whole mount *in situ* hybridization (Fig. 4*A*). Specific staining was found beginning with embryonic stage 9. In stages 9 and 10, *ninaD* expression occurred in the midgut primordia and in mesodermal cells, from which hemocytes and macrophages as well as fat body cells derive. In the embryonic stages 11–14, a more punctuated

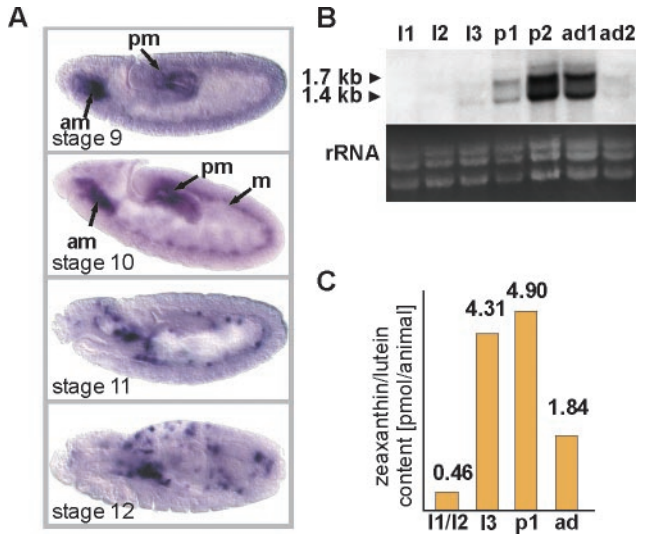

Fig. 4. *ninaD* gene expression during different developmental stages. (*A*) Whole mount *in situ* hybridizations of *wt* embryos by using a digoxygenin-labeled *ninaD* antisense riboprobe. Lateral view of stained embryos of different developmental stages. In stage 9 and 10 embryos, *ninaD* is expressed in the anterior and posterior midgut primordia as well as in the mesoderm. Beginning with stage 11, the staining of coherent tissues disappears, and *ninaD* expression can be observed in individual large cells resembling hemocytes both in their size and distribution. From a center close to the anterior midgut primordium, the cells appear to migrate anteriorly and posteriorly along the mesoderm. In stage 12, the stained single cells are more randomly distributed. am, anterior midgut primordium; m, mesoderm; pm, posterior midgut primordium. (*B*) Northern blot analysis of *ninaD* mRNA levels in different postembryonic developmental stages of *wt* flies. l1–l3, first to third instar larvae; p1 and p2, first and second pupal stage; ad1, imagoes (1–2 days); ad2, imagoes (>10 days). (*C*) Zeaxanthin and lutein contents of the different postembryonic stages as determined by quantitative HPLC analysis. ad, 1- to 6-day-old imagoes. The values give the average of 50 flies per experiment.

staining could be observed resembling that described for hemocytes. Interestingly, expression in hemocytes has also been reported for the *Drosophila* CD36-like scavenger receptor encoded by the gene *croquemort* (27) and is characteristic for mammalian class B scavenger receptor counterparts like CD36 (24).

By Northern blot, we then determined the postembryonic *ninaD* expression and compared it to the carotenoid contents from the different developmental stages (Fig. 4 *B* and *C*). The *ninaD* mRNA level rose at larval stage 3 (L3) and was strongest in the pupal stages. After eclosion, the *ninaD* mRNA level remained high for 1 to 2 days but was lower in older imagoes (>10 days). The increase of the *ninaD* mRNA level in the late larval stages corresponded to some extent with the accumulation of carotenoids found here. In pupae, the carotenoid content was still high but decreased in young imagoes, evidently correlated with the synthesis of the visual chromophore during metamorphogenesis. The high *ninaD* mRNA level in the pupae might indicate that the *ninaD* scavenger receptor is of particular importance for the redistribution of the carotenoids from larval stores to the developing eyes. To address the role of the *ninaD* scavenger receptor in mediating the cellular uptake of carotenoids into any target tissues, we placed the UAS-$ninaD^{(wt)}$ transgene under the control of *ninaE*-GAL4. The $ninaD^{(wt)}$ transgene expression should then result in a specific high level expression in the R1-R6 photoreceptor cells and thus in carotenoid accumulation in the fly's head. Indeed, by quantitative HPLC analysis of the carotenoids of the heads of these transgenic flies, a 2.5-fold level in zeaxanthin and lutein was found compared with *wt* controls.

Discussion

In animals, it is long known that carotenoids exert a variety of physiological functions, but the molecular players involved in their absorption and body distribution have not yet been identified. We now show that the molecular basis of the blindness of the *Drosophila ninaD* mutant is a nonsense mutation in a gene encoding a lipid class B type scavenger receptor, leading to a defect in carotenoid utilization for the synthesis of visual chromophore. The identity of the *ninaD* gene with this class B scavenger receptor was confirmed by *P* element-mediated rescue with a $ninaD^{(wt)}$ scavenger receptor allele. Its heat shock-induced expression restored carotenoid uptake and visual pigments in the $ninaD^{P245}$ mutant. Furthermore, ectopic UAS-$ninaD^{(wt)}$ expression under control of a *ninaE*-GAL4 driver specifically led to an elevation of the carotenoid content in the fly's head. Thus, these analyses provide genetic and functional evidence that class B scavenger receptors are causally involved in the cellular uptake of carotenoids into cells of target tissues.

ninaD belongs to a small family of *Drosophila* class B scavenger receptor-like proteins. Two other representatives, encoded by *croquemort* and *emp*, have so far been characterized (27, 28). For *croquemort*, expressed in embryonic macrophages, a crucial role in the recognition and phagocytosis of apoptotic cells during *Drosophila* embryonic development has been recently demonstrated (27). Comparable functions of scavenger receptors have also been reported in mammals (31). The physiological functions of the *emp* scavenger receptor have yet not been addressed in detail.

In the past few years, there has been growing evidence that class B scavenger receptors, in particular SR-BI, are substantially involved in lipid metabolism, especially the cholesterol homeostasis in mammals (32, 33). It could be shown that these receptors mediate the bidirectional flux of unesterified cholesterol between target cells and the circulating lipoproteins (34, 35). Yet, a direct involvement of class B scavenger receptors in insect lipid metabolism has not been reported. In insects, carotenoids are transported in the lipophorins of the hemolymph (36). These lipophorins are structurally related to the mammalian lipoprotein particles, also being transport vehicles for carotenoids (6). Insect *in vitro* systems provided evidence that the cellular uptake of lipids occurs by a flux between lipophorins and target cells. Like cholesterol exchange in mammals, the lipophorin particles are not necessarily internalized by receptor-mediated phagocytosis (37, 38). Based on our analyses, we speculate that the *ninaD* scavenger receptor mediates the carotenoid transport from lipophorins in a mechanistically similar manner.

ninaD mRNA levels were particularly high in pupae when lipids acquired during the larval stage are mobilized and redistributed for metamorphogenesis. The high mRNA levels during this developmental stage may indicate that NinaD plays a specific role for the redistribution of xanthophylls, the pupal storage form of carotenoids (14). This mobilization is necessary to deliver carotenoids from adipose tissues to the developing eyes for the synthesis of the visual chromophores. Because *ninaD* flies develop normally and possess, besides reduced carotenoids, no significant alterations in their lipid contents, the loss of this scavenger receptor seems not to interfere with the fly's lipid metabolism in general. This situation may be explained by the specificity of the NinaD function for carotenoids or by a bypass of the lack of NinaD function by different, yet to be identified receptor types.

With the molecular identification of the *Drosophila* NinaD and NinaB functions, the two crucial molecular players involved in the synthesis of the visual chromophore from dietary carotenoid precursors are now molecularly identified in this model organism. As for mammals, we already showed that the ortholog of

NinaB, a β,β-carotene-15,15′-oxygenase, catalyzes β-carotene conversion to vitamin A (10). In mammals, the NinaB ortholog is expressed in a variety of different tissues, including the eyes and reproductive organs (10, 12, 13), so vitamin A-dependent physiological processes like vision and reproduction may be directly influenced by carotenoids, which then are tissue specifically acquired from lipoproteins of the circulation. It remains to be shown whether the cellular uptake of carotenoids necessary for their conversion to vitamin A is mediated via the mammalian NinaD receptor counterparts. Vitamin A also plays an important role in the mammalian immune response (39) and a role of scavenger receptors in host defense has been well established (40). Therefore, a putative role of class B scavenger receptors in provitamin A uptake may interlink some aspects of vitamin A and class B scavenger receptor functions in the immune system. Furthermore, it has recently been reported that in SR-BI-deficient mice, vitamin E metabolism is impaired, resulting in an elevated plasma concentration of this fat-soluble vitamin (41). This finding, together with our results from *Drosophila*, indicates a more general role of class B scavenger receptors in the metabolism of fat-soluble vitamins belonging to the isoprenoid substance class. No defects related to carotenoid utilization have as yet been reported in mice deficient in the class B scavenger receptors, SR-BI or CD36. However, it should be noted that under laboratory conditions mice are usually kept on a diet rich in preformed vitamin A. Therefore, an impaired carotenoid utilization would not necessarily result in vitamin A deficiency accompanied by phenotypic alterations such as blindness.

Besides vitamin A formation, carotenoids are of importance for various physiological processes. For example, lutein and zeaxanthin are accumulated as macular pigments in the primate and human eye (42). Additionally, beneficial effects of carotenoids mainly due to their antioxidant properties have been discussed in the context of several diseases (43). The identification of the scavenger receptor *ninaD* as being essential for carotenoid uptake in *Drosophila* delivers molecular insight into how dietary carotenoids may be distributed to their site of action in the body and promises to elucidate new aspects of class B scavenger receptor functions.

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